

Biomaterials by Design: Layer-By-Layer Assembled Ion-Selective and Biocompatible Films of TiO₂ Nanoshells for Neurochemical Monitoring**

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Titania nanoshells with an external diameter of 10–30 nm and a wall thickness of 3–5 nm were prepared by dissolving the silver cores of Ag@TiO₂ nanoparticles in a concentrated solution of ammonium hydroxide. The nanoshells were assembled layer-by-layer (LBL), with negatively charged poly(acrylic acid) (PAA) to produce coatings with a network of voids and channels in the interior of the film. The diameter of the channels in the titania shells was comparable to the thickness of the electrical double layer in porous matter (0.3–30 nm). The prepared nanoparticulate films demonstrated strong ion-sieving properties due to the exclusion of some ions from the diffuse region of the electrical double layer. The permeation of ions could be tuned effectively by the pH and ionic strength of a solution between “open” and “closed” states. The ion-separation effect was utilized for the selective determination of one of the most important neurotransmitters, dopamine, on a background of ascorbic acid. Under physiological conditions, the negative charge on the surface of TiO₂ facilitated the permeation of positively charged dopamine through the LBL film to the electrode, preventing the access of the negatively charged ascorbic acid. The deposition of the nanoshell/polyelectrolyte film resulted in a significant improvement to the selectivity of dopamine determination. The prepared nanoshell films were also found to be compatible with nervous tissue secreting dopamine. Although the obtained data demonstrated the potential of TiO₂ LBL films for implantable biomedical devices for nerve tissue monitoring, the problem of electrode poisoning by the by-products of dopamine reduction has yet to be resolved.

1. Introduction

Nanoparticles composed of various materials have attracted tremendous attention in the research community, due to their unique size-dependent properties,^[1] which originate from the large contribution of surface atoms to the properties of nano-

scale objects and from the size quantization effect.^[2,3] Although the majority of current work on nanoparticles is focused on their optical, electrical, and magnetic properties and the corresponding devices,^[4–10] a new field of biomedical applications of semiconductor and metal nanoparticles has begun to emerge.^[11] For instance, II–VI semiconductor and gold nanoparticles modified with antibodies or oligonucleotides can be used as highly stable luminescent and colorimetric tags for immunoanalysis.^[11] Magnetic nanoparticles are being tested as contrast agents for cancer imaging and treatment.^[12] BiI₃ nanoparticles have been proposed for the preparation of X-ray sensitive films for digital X-ray radiology.^[13]

In this work, we were interested in expanding the scope of possible biomedical applications of nanoparticles and, in particular, we found that nanoparticle films may be utilized for neurochemical monitoring. This has become possible due to the utilization of a new type of nanoparticles—titania nanoshells—with a morphology of hollow spheres 10–30 nm in diameter. Unlike previously made nanostructured forms of titania,^[14] nanoshells of this size enable selective ion permeation because their thin films have voids and channels of different origin, with pores in the walls of the shells being one of the structural elements. The diameter of the pores is comparable to the thickness of the electrical double layer, thus leading to ion-sieving properties.

A colloidal dispersion of nanoshells was processed to form thin films suitable for electrode coatings using layer-by-layer assembly (LBL) with polyelectrolytes.^[15–18] Investigating the structure and ion transport through the LBL nanoshell films,

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[**] N.A.K. thanks NSF-CAREER (CHE-9876265), AFOSR (F49620-99-C-0072), NATO (#CRG 971167), OSU Sensor Center, and Nomadics Inc. for the partial financial support of this research. D.S.K. acknowledges support from the NSF/NATO (NSF/NATO Grant #DGE-9902637) for the postdoctoral fellowship. L.M.L.M. acknowledges financial support from the Spanish Xunta de Galicia, (Project no. PGIDT99PXI30104B), Ministerio de Educación y Cultura (Project no. PB98-1088), and NATO (#CRG 971167). I.P.S. thanks the Spanish Ministerio de Educación y Cultura for an FPI fellowship.

we found that they are capable of separating one of the most important neurotransmitters, dopamine, from ascorbic acid. Currently, the lack of dopamine/ascorbic acid selectivity for electrochemical in-vivo detection of dopamine presents a significant problem for monitoring brain activity, and for diagnosing Parkinson's and similar neurological diseases.^[19,20] The utilization of nanoshell coatings enables a significant improvement in dopamine selectivity, by suppressing the interference from oppositely charged electroactive ions. We also demonstrate that these films are biocompatible with cultured PC12 cells, a model line for nerve cells, which successfully attach to, and grow on, the film's surface. The combination of dopamine selectivity and biocompatibility is essential for the design of long-term implantable neurological devices and for monitoring the activity of neuron cells.

2. Results

2.1. Preparation of Titania Nanoshells

Hollow titania spheres were synthesized from composite Ag/TiO₂ nanoparticles, which are made of a silver core with a thin TiO₂ layer around it. The core-shell morphology of the precursor particles is clearly visible in the transmission electron microscopy (TEM) image (Fig. 1a). The Ag/TiO₂ colloids are approximately spherical in shape, and the diameter of most of the particles lies between 10 and 30 nm.

In order to make nanoshells, the silver core was removed by adding a concentrated ammonia solution. NH₃ molecules form coordinate compounds with Ag surface atoms, thus reducing the redox potential of the Ag/Ag⁺ pair and resulting in the oxidation of silver metal by oxygen from the environment. The oxidation products, i.e., silver ion complexes, diffuse out from the interior of the Ag/TiO₂ nanoparticles into the solution through the pores in the titania shell.^[21] Upon complete extraction of the silver core, ammonia treatment yields hollow, porous spheres of TiO₂ with a shell thickness of 3–5 nm, as evidenced by TEM (Fig. 1b).

The X-ray photoelectron spectroscopy (XPS) signal contains cumulative information on the chemical composition of a large number of particles that might not have been surveyed by TEM. Therefore, this technique was used to confirm the completeness of core removal in all precursor nanoparticles. The peaks at 374 and 369 eV, assigned to the 3d³ and 3d⁵ levels of the Ag core, completely disappear after ammonia treatment (Figs. 2a,b, inset). Other strong peaks are present in both the Ag@TiO₂ and the nanoshells, and these correspond to titanium (464 and 459 eV), oxygen (531 eV), silicon (102 eV; from the silicon wafers used as XPS substrates), and carbon (286, 269, and 198 eV; from the polymer).

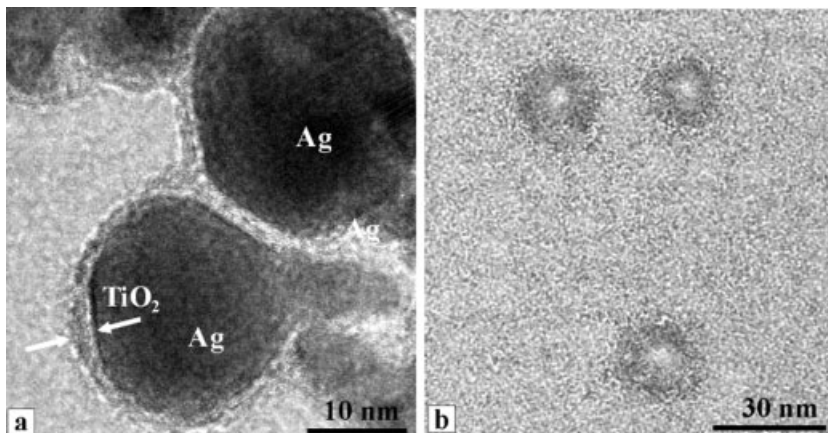


Fig. 1. Transmission electron microscopy images of a) Ag-core/TiO₂-shell nanoparticles; b) TiO₂ nanoshells.

2.2. Preparation and Structure of TiO₂ Nanoshell Films

For the preparation of thin films of titania nanoshells, we utilized the LBL technique. This technique was recently introduced into practice by Decher, Möhwald, and co-workers for polyelectrolyte pairs, for which it has found many implementa-

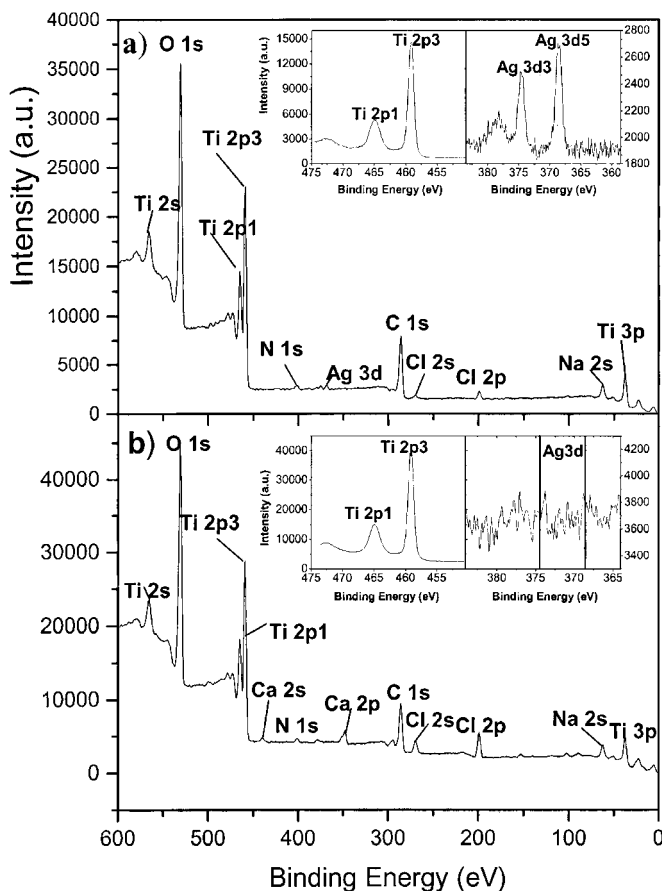


Fig. 2. High-resolution X-ray photoelectron spectra of Ag-core/TiO₂-shell nanoparticles: a) with a silver core; b) without a silver core. Insets show the (magnified) Ti 2p and Ag 3d regions.

tions.^[16,22,23] LBL assembly can be described as the sequential dipping of a substrate into solutions/dispersions of oppositely charged species. It exploits the same idea as the layering method first used by Iler.^[24] Similar approaches to making thin films have also been used by Bruce et al.^[25] for magnetic nanoparticles, and Nicolau et al.^[26,27] for semiconductor thin films. Subsequent studies have revealed that LBL could be successfully utilized for making thin films from many nanoparticles and other inorganic colloids.^[17,28–34]

TiO₂ nanoshells form a stable dispersion at acidic pH, when they are positively charged (point of zero charge (pzc) of TiO₂ is ca. pH 6). At pH 2, the zeta potential of the nanoshell dispersion was determined to be +30 mV. Hence, a negatively charged polyelectrolyte, such as poly(acrylic acid) (PAA), could be employed to prepare LBL films from the composite core-shell nanoparticles, as has been previously demonstrated.^[35] The sequential adsorption of PAA and TiO₂ nanoshell layers results in a gradual build-up of a composite film, which can be evidenced by a variety of experimental techniques such as ellipsometry (Fig. 3); the growth of the film for the composite TiO₂-on-Ag nanoparticles has

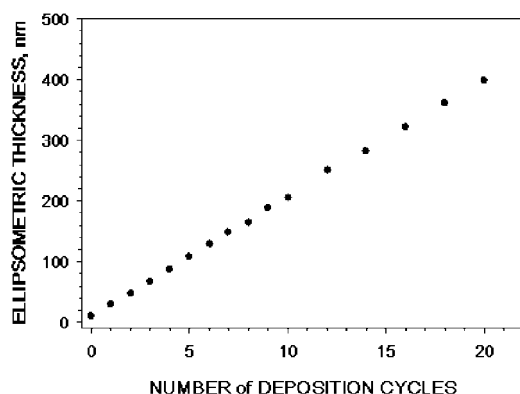


Fig. 3. Ellipsometric measurements of the thickness of a multilayer TiO₂ film vs. the number of adsorption cycles.

been studied by UV-vis absorption spectroscopy—details are given in our previous publication.^[35] The film build-up occurred over a fairly wide range of conditions. In this study, we typically used the films obtained at pH 2.5, which displayed a dense packing of the composite TiO₂-on-Ag nanoparticles, and which were subsequently converted into the nanoshells as described in the Experimental section. Atomic force microscopy (AFM) and scanning tunneling microscopy (STM) images show the in-plane structure of the assembled nanoshell layers (Fig. 4). STM images, which typically give a better resolution than AFM, demonstrate the closely packed structure of the film (Fig. 4b): the AFM image taken over a larger surface area shows the uniformity of the packing (Fig. 4a). A dense and uniform packing of the nanoparticle film is critical for attaining selective ion transport.^[36,37] No difference in particle packing and image features was observed before or after removal of the silver core.

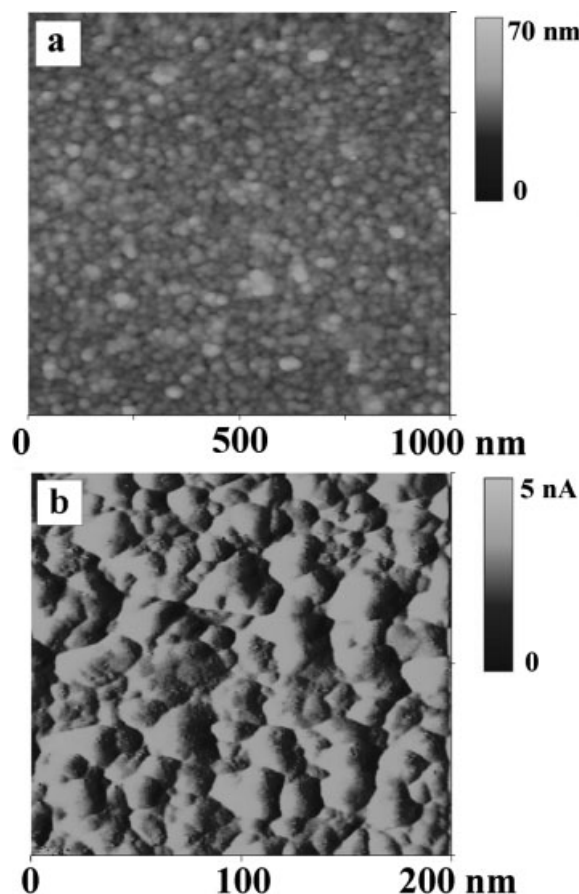


Fig. 4. a) AFM and b) STM images of a monolayer of TiO₂ nanoshells assembled on a gold surface.

2.3. Ion-Sieving Properties

The existence of pores in the TiO₂ nanoshells that are large enough to allow [Ag(NH₃)₂]⁺ ions to escape is one reason to expect a sufficient ion permeability in LBL films made from them. The hollow sphere geometry of the nanoshells with thin walls provides both a high surface area and a nanoscale porosity. All these structural features are indicative of ion-sieving capabilities of the nanomaterial.^[38] Therefore, we investigated the ion-permeable properties of the nanoshell films using the standard electrochemical method of cyclic voltammetry, with potassium ferricyanide, [Fe(CN)₆]^{3−}, as the probe molecule.^[39–46] This probe was selected on the basis of its formal redox potential, which must be within the stability limits of the films. Figures 5a and c show the voltammograms obtained on an LBL coated glass carbon (GC) electrode *with* and *without* Ag cores. Figure 6 displays the dependence of the peak current on the sweep rate. Both of these sets of data are necessary in order to evaluate the mechanism of ion transport through the nanoshell LBL film.

The ion permeability of the LBL nanoshell films strongly depends on the pH and ionic strength (Figs. 5, 6). The flow of [Fe(CN)₆]^{3−} from the solution to the electrode is almost com-

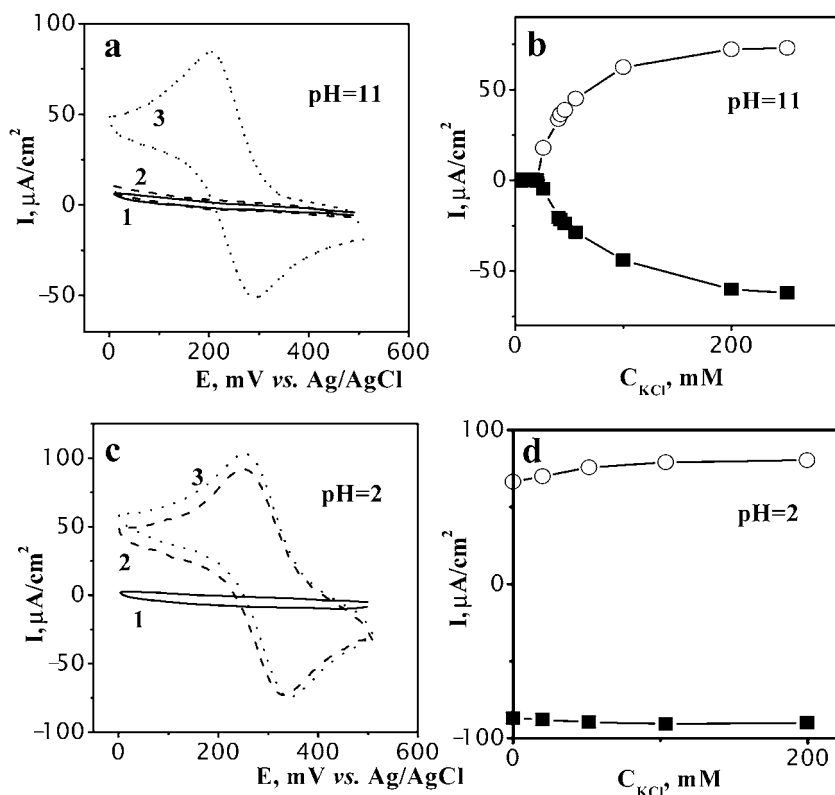


Fig. 5. Cyclic voltammetry data in 2 mM $\text{K}_3[\text{Fe}(\text{CN})_6]$, on a glassy carbon electrode coated with a PDDA(PAA/TiO₂)₂₀ film. The data were taken at: a,b) pH 11; c,d) pH 2. On (a) and (c), the individual traces are labeled (1–3) and correspond to: 1) electrodes coated with Ag/TiO₂ core-shell particles; and to a nanoshell-modified glassy carbon electrode in 2) 0.02 M KCl; and 3) 0.5 M KCl. Graphs (b) and (d) show the plots of the cathodic (■) and anodic (○) maximum cyclic voltammetry peak currents vs. the concentration of supporting electrolyte. The scan rate was 5 mV/s.

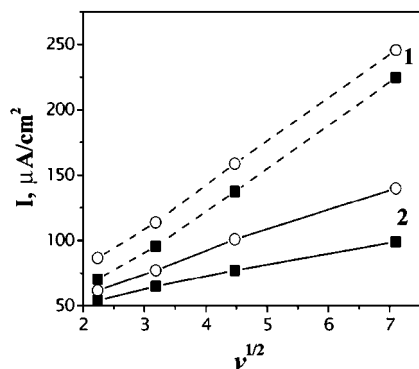


Fig. 6. Scan rate dependence of the cathodic (■) and anodic (○) peak currents vs. $v^{1/2}$ for an electrode modified with a PDDA(PAA/TiO₂)₂₀ film in a 2 mM $\text{K}_3[\text{Fe}(\text{CN})_6]/0.5$ M KCl solution at different pH values: 1) pH 2 and 2) pH 11.

pletely blocked at low ionic strength and at pH 11 (Fig. 5a, trace 2 and Fig. 5b). At the same time, the ionic strength has little effect when the nanoshell film is at pH 2 (Fig. 5c, traces 2, 3 and Fig. 5d). The change of pH from 11 to 2 results in a drastic increase in the transparency of the nanoshell film toward the probe molecule, as indicated by the increase in the peak current in the voltammograms (Figs. 5, 6).

To understand better the origin of the ion-sieving properties in the LBL films, it is necessary also to look into the same de-

pendencies for solely polyelectrolyte films without the nanoporosity introduced by nanoshells. The multilayer assembly made of poly(diallyldimethylammonium chloride) $M_w = 400\,000$ – $500\,000$ (PDDA) and PAA also revealed a similar effect of ionic strength and pH in cyclic voltammetry experiments with $[\text{Fe}(\text{CN})_6]^{3-}$ (Fig. 7). The degree of blocking of ionic current was lower for low ionic strength and high pH, as was the case for the nanoshell composite film, as indicated by the non-zero current at pH 11 when the concentration of KCl approached 0.0 M.

2.4. Separation of Dopamine

The small thickness, of the order of hundreds of nanometers, and the high density of pores per unit volume—characteristic of TiO₂ nanoshell LBL films as well as at least some polyelectrolyte LBL multilayers^[47]—resemble the qualities of biomembranes and tissues produced by biomineralization.^[48] In a similar way to polyelectrolyte/TiO₂ films, biomembranes also combine organic and inorganic components.^[49,50] This resemblance prompted us to seek biological functionalities of the nanoparticle LBL films and the observed ion-selective properties.

In recent years, there has been considerable interest in developing detection methods for the secretion of small molecules, known as neurotransmitters, from cells deep inside the brain.^[19,20] Understanding the mechanism of their secretion and metabolism is the key to curing many neurological disorders, such as Parkinson's disease, as well as drug addiction. Among them, dopamine is one of the most important chemicals in the nervous system. It controls movement, emotional response, and the ability to experience pleasure and pain. Dopamine can easily be detected electrochemically with a carbon fiber electrode.^[42] However, one of the major problems with electrochemical detection is the interference of ascorbic acid, which is often present with dopamine in tissues, and has virtually identical electrochemical behavior.^[51]

The blocking of the permeation of negatively charged $[\text{Fe}(\text{CN})_6]^{3-}$ ions (Figs. 5–7) suggests the possibility of imparting a selectivity to dopamine over ascorbic acid, which forms negatively charged ions. Although these substances are both electroactive and are similar in size, the film structure and conditions may be chosen so that the permeation of ascorbic acid is blocked. In order to test this hypothesis, the electrochemical response of LBL-modified electrodes to dopamine and ascorbic acid was compared with that of uncoated electrodes (Figs. 8–10). For this, we used differential pulse voltammetry to monitor the electrode reactions: this is the primary method of dopamine detection in neurochemistry. It can be seen that the LBL coating with nanoshells makes a drastic difference to the

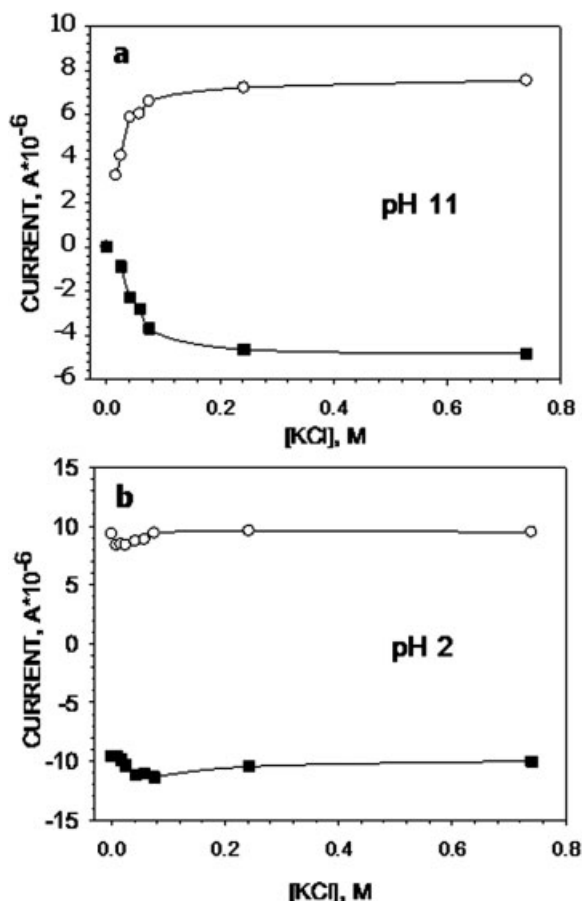


Fig. 7. A plot of the cyclic voltammetry cathodic (■) and anodic (○) peak currents in a cyclic voltammetry experiment in 2 mM K₃[Fe(CN)₆] on a glassy carbon electrode coated with a (PDDA/PAA)₂₀PDDA film vs. the ionic strength of the supporting electrolyte. The data were taken a) at pH 11 and b) at pH 2. The scan rate was 5 mV/s.

magnitude of the electrochemical signal of ascorbic acid relative to dopamine (Fig. 8). Without this modification, 1 mM ascorbic acid produces a peak (Fig. 8a, trace 2), which is three times stronger than that of an equal (1 mM) concentration of dopamine (Fig. 8a, trace 1). The peak arising from the 1:1 molar mixture of dopamine and ascorbic acid (Fig. 8a, trace 3) is approximately equal to the superposition of those obtained separately, with the contribution from ascorbic acid being dominant. When the electrode was coated with 20 LBL layers of TiO₂ nanoshells, the peak from ascorbic acid became barely visible (Fig. 8b, trace 2), whereas that of dopamine (Fig. 8b, trace 1) significantly increased, as compared to that on the bare electrode.

The (PDDA/PAA)₂₀PDDA polyelectrolyte LBL films made after the same number of deposition cycles as for nanoshell/PAA also exhibit a selectivity for dopamine, while blocking the permeation of ascorbic acid (Fig. 9). The effect is comparable to that for nanoshell/PAA films, but the greater background current and stronger interference from some side processes should be noted.

An important aspect for the long-term neurochemical monitoring of dopamine is the prevention of electrode poisoning. A comparison of the native GC surface and nanoshell/PAA- and PDDA/PAA-modified electrodes can be made on the basis of consecutive pulse voltammetry curves obtained on the same electrode one by one (Fig. 10). Apparently, neither nanoshell/PAA- nor PDDA/PAA-coated electrodes differ significantly from the naked electrode in maintaining the quality of the electrode surface, and the initially high transparency of the coating quickly degrades during the course of dopamine reduction. For the PDDA/PAA-coated electrodes, an additional poisoning reaction reveals itself in the second peak at 370 mV, in the later voltammograms.

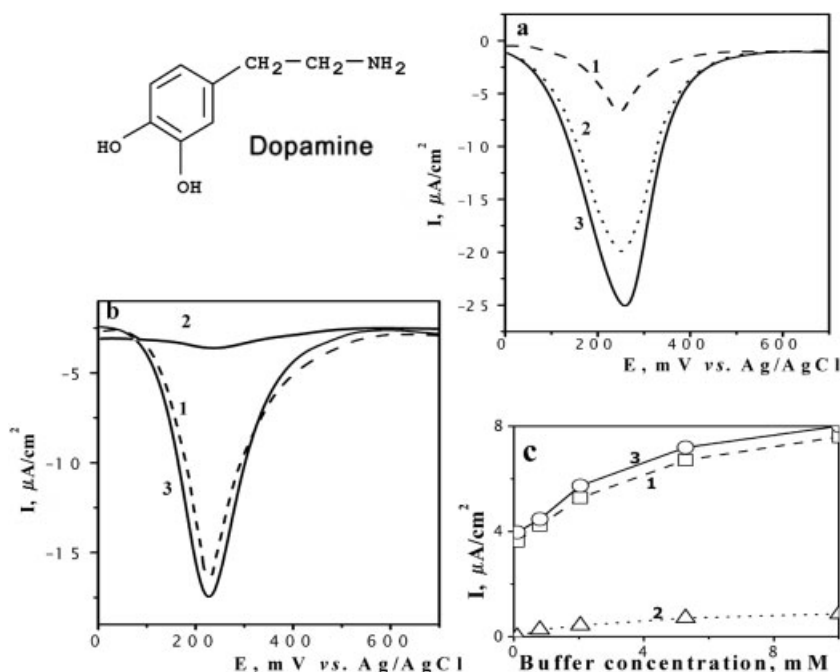


Fig. 8. Differential pulse voltammograms for 1: 1 mM dopamine; 2: 1 mM ascorbic acid; and 3: a mixture of 1 mM dopamine + 1 mM ascorbic acid in 0.1 M phosphate buffer for: a) a nascent electrode and b) an electrode coated with PDDA(PAA/TiO₂)₂₀. c) A plot of the peak currents of the differential pulse voltammograms for 1: 1 mM dopamine; 2: 1 mM ascorbic acid; and 3: a dopamine/ascorbic acid mixture; vs. the concentration of phosphate buffer.

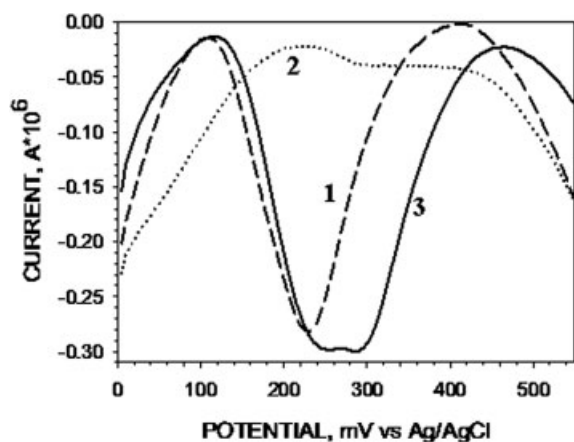


Fig. 9. Differential pulse voltammograms for 1) 1 mM dopamine; 2) 1 mM ascorbic acid; and 3) a mixture of 1 mM dopamine + 1 mM ascorbic acid in 0.1 M phosphate buffer; for a glassy carbon electrode coated with a PDDA(PDDA/PAA)₂₀ film.

2.5. Biocompatibility

For any biomedical application, the demonstrated ion-selective functionality should be complemented by biocompatibility. Unlike an electrochemical cell, the brain is a biologically active environment. The implantation of an electrode is traumatic to brain cells, and can cause a rejection reaction, or the death of the surrounding tissue: both of which must, of course, be avoided. Figure 11 demonstrates the successful attachment of the neuron precursor PC12 pheochromocytoma culture cells to a petri dish coated by the PAA/nanoshell multilayers. The attachment of the same culture cell was not observed for PAA/PDDA multilayers and is, therefore, not shown.

3. Discussion

3.1. Film Structure

In addition to an array of optical, magnetic, electrical, and other advanced coatings, recent studies indicate that LBL offers a powerful and technologically convenient tool for the preparation of biomaterials.^[47,52–56] Among them, organic–inorganic composite thin films have the advantage of functional versatility, better overall mechanical properties, and improved biocompatibility, as compared to pure inorganic materials.^[57] Furthermore, the LBL nature of the deposition offers better overall structural control of the coating than do other thin film methods based on bulk composite preparation.^[16,58]

At the same time, LBL deposition also introduces its own challenges, and one of these is the attainment of a high surface density of nanoparticles.^[59] It needs to be emphasized that, for any electrochemical applications of nanoparticle LBL films, good lateral packing is of great importance, although it is sometimes hard to achieve.^[18] In order to control the film's structure, one can vary several experimental parameters, such as exposure time, concentration of reagents, ionic strength, pH,

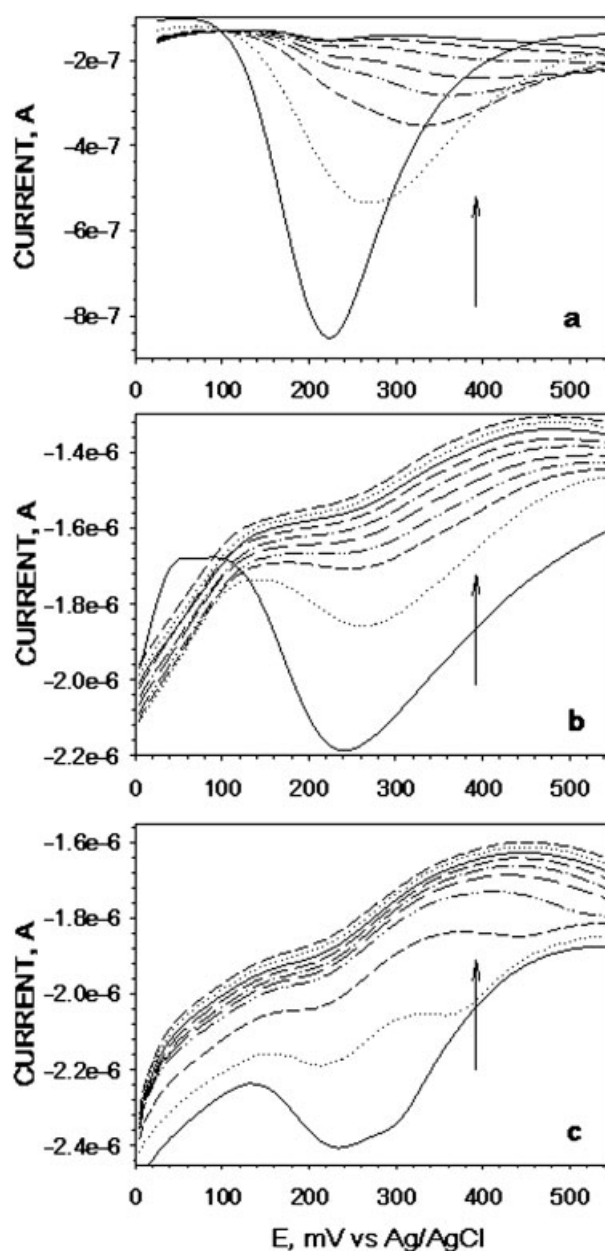


Fig. 10. Differential pulse voltammograms obtained on the same electrode in the course of consecutive repetition of the voltage scan in the presence of 1 mM dopamine: a) nascent glassy carbon electrode; b) glassy carbon electrode coated with a PDDA(PAA/TiO₂)₁₅ film; c) glassy carbon electrode coated with a (PDDA/PAA)₂₀PDDA film. The arrows indicate the progression in which the scans were obtained, from the first (bottom) to the last (top).

and surface composition. Assembly conditions where both repulsion and attraction are strong promote well-ordered, closely packed structures that are determined by both electrostatic and van der Waals interactions.^[60–62] For LBL assembly of titania nanoshells, we utilized pH tuning, which changes the entire spectrum of ionic and non-ionic interactions and, therefore, is quite efficient.^[59] By systematic AFM imaging of films obtained after one deposition cycle, i.e., PDDA/PAA/TiO₂, the best ordering and packing of the nanoshells on PAA was observed at pH 2.5 for TiO₂ dispersions, pH 3.5 for PAA solutions, and pH 3.5 for PDDA (first “basement” layer) (Fig. 4).

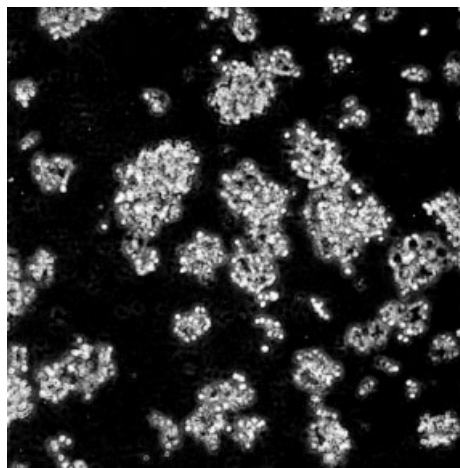


Fig. 11. Light 100 $\times$ microscopy image of PC12 cultured cells on substrate-coated PDDA(PAA/TiO₂)₅ after 24 h of exposure to the cell culture at 37 °C.

For comparative studies of electrochemical properties, the solely polyelectrolyte LBL films (PDDA/PAA)_{*n*} were assembled under the same conditions.

3.2. Ion-Sieving Mechanism

The magnitude of the voltammetric redox currents—both cathodic and anodic—reflects the barrier properties of the coatings. Despite the conductive nature of the silver core, the voltammograms obtained using the electrode covered by Ag-core/TiO₂-shell nanoparticles indicate that the multilayer film from the precursor nanoparticle *with* a silver core effectively passivates the electrode surface and blocks the oxidation and reduction of the probe at any pH, and at any ionic strength (Figs. 5a and c, trace 1). Conversely, when the silver cores are removed, a strong current from the redox reactions of [Fe(CN)₆]³⁻ is observed (Figs. 5a and c, trace 3). The linear dependence of *I* on $\nu^{1/2}$ over a wide range of pH (Fig. 6) proves that the observed electrochemical processes are diffusion controlled, which is commensurate with the through-pore mass transfer mechanism of [Fe(CN)₆]³⁻ that is typical for many membranes. The permeability of these layers without the silver core shows that the penetration of ions occurs predominantly through the shell rather than through the possible gaps between the TiO₂ hollow spheres or defects in the coating. For poorly packed films, a substantial background current was observed before and after removal of the core. The prevention of defect currents necessitated maximization of the packing density of the nanoshells in the film, and pH optimization (Fig. 4).

The structural information obtained about the films and nanoparticles helps to understand the mechanism of ionic selectivity and transport. Importantly, the ion permeability curves for PAA/TiO₂ and PAA/PDDA films are very similar (Figs. 5b,d and 7). In addition, the nature of the terminal layer—positive or negative—did not appear to have an effect on the voltammograms. These observations give evidence that the Donnan equilibrium, which was considered to be the pri-

mary source of ion selectivity in polyelectrolyte LBL assembled systems,^[63,64] is not the reason for ionic sieving in this system. The observed on/off behavior of the ion diffusion (Fig. 5) in response to the changes in pH and ionic strength is related to the distribution/organization of charges in the *bulk* of the assembled film, i.e., the behavior is based on electrostatic (and possibly chemical) interactions with the film matrix.

The molecular mechanism of the pH/ionic strength dependence of ion permeability can be understood by considering the model of ions/double layer interactions inside ultra-narrow pores, as suggested by Martin and co-workers^[65–68] and further developed by Crooks and Bruening and their co-workers.^[39–46,69] For channels that have a diameter comparable to the thickness of the diffuse part of an electrical double layer, i.e., 0.3–30 nm, the Hemholtz layers on the pore walls may overlap. When this happens, the ions of one sign tend to dominate in the channel, while their counterions are virtually expelled from it. This results in the exclusive transport of the ions with the dominant charge and the stoppage of the minority charge carriers. The selectivity of the membrane improves with lowering ionic strength, when the diffuse layers become thicker, and worsens for the opposite trend. Changing the pH may reverse the charge of the electrical double layer and, thus, the roles of counterions and their relative selectivity.^[39,46,65]

The behavior of the TiO₂ nanoshell films can be adequately described by this model. The variation of the surface potential of TiO₂ with ionic strength and pH is well known.^[70,71] For the LBL films of nanoshells at pH 11, the diffuse part of the electrical double layer is formed predominantly by positively charged ions of the supporting electrolyte (K⁺; Figs. 5a,b). Negatively charged [Fe(CN)₆]³⁻ ions are mostly excluded from the network of voids and channels formed by nanoshells and, therefore, the redox current is virtually zero in 0.02 M KCl (Figs. 5a,b). When the ionic strength is increased to 0.5 M KCl, the diffuse part of the double layer shrinks, opening the space for diffusion of the negatively charged ions, and the current increases (Fig. 5b). At low pH, the electrical double layer is always formed from the negatively charged ions of [Fe(CN)₆]³⁻ and Cl⁻ and, therefore, their diffusion through the film is quite efficient for any ionic strength (Figs. 5c,d).

Complete closing of the nanoshell film occurs when the concentration of KCl is 0.02 M. The characteristic thickness of the electrical double layer according to the Gouy–Chapman theory under these conditions is 2–2.5 nm,^[72,73] which indicates that the upper limit of the pore diameter is 4–5 nm. The existence of pores in this size range in the nanoshells can be seen in the AFM images obtained with high aspect ratio AFM probes (Fig. 12a). Similar openings in the shells can also be seen in the TEM images (Fig. 12b).

PAA, the second major component of the TiO₂ nanoshell LBL films, is likely to participate in the closing and opening of the ion channels as well. In the framework of the model presented above, pores in the films may form due to the pH-dependent change of film morphology, although this process is likely to be inherently slow and, at least to some extent, irreversible because of the high molecular weight of the polymer.^[74–77] It is more likely that areas of spontaneous accumula-

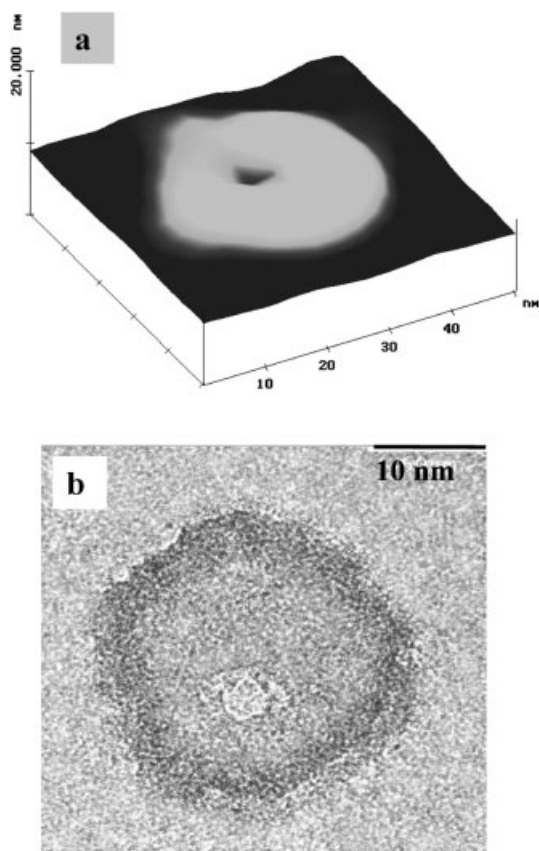


Fig. 12. Images of nanoshells with pores: a) AFM image and b) TEM image.

tion of PAA chains, resulting from the accidental distribution of topographic features on the surface or coiled conformation of the polyelectrolyte, act as ion-sieves. However, the actual structure of molecular associates that constitute the nanometer scale ion channels in polyelectrolyte/polyelectrolyte LBL films is difficult to establish. The Donnan equilibrium may also play a role in the ion separation in solely electrolyte films, as was demonstrated by Krasemann and Tieke^[64] and Bruening and co-workers,^[63,78] although it is supposed to be more pronounced for doubly and triply charged ions than for the singly charged ascorbic acid and dopamine.

It is important to emphasize the significance of the nanoshell morphology of the particles in obtaining the pronounced ion-selective properties in the LBL film. The shells provide rigid pores with oxide walls comparable in dimension with the thickness of the electrical double layer, which is absent in solid particles. Simultaneously, the 10–30 nm scale particles form layers with few defects above that limit, which would be difficult to achieve with particles in, for instance, the 10 nm range. Defects of this dimension would create a significant background current, masking the ion conductance through the pores. This also underscores the importance of fine-tuning the deposition conditions of the nanoshells, maximizing the particle density of the surface. We also noticed that only freshly prepared nanoshells displayed the ion-selective behavior described, whereas the colloid assembled after a 3 month aging period, under exactly the same conditions, showed a significantly worse performance,

presumably because of unoptimized nanoshell packing, which no longer corresponds to the altered balance of attractive/repulsive forces caused by the change of charge distribution on the nanoshell surface.^[35,60]

3.3. Permeation of Dopamine

Considering the predominantly electrostatic nature of the ion-sieving effects in porous LBL films, one can take advantage of the difference in the ionization state and the characteristic charge of the permeating ions (e.g., dopamine and ascorbic acid). Provided a sufficient space charge exists inside the nanoparticle channels, an electrode coated with a LBL layer of nanoshells may behave as if it is simultaneously in the “open” state for dopamine and in the “closed” state for ascorbic acid. This makes possible the selective enhancement of the permeation of positively charged dopamine and retardation of the transport of negatively charged ascorbic acid.

All measurements were made at pH 7. At this pH, TiO₂ nanoshells are negatively charged and, therefore, the diffuse part of the double layer is made primarily from cations, as is required for the separation of positively charged dopamine and negatively charged ascorbic acid. The ratio between the dopamine and ascorbic acid signals changes from 1:3 for a native GC surface to 9:1 for a nanoshell-modified electrode, which gives an overall 27-fold enhancement of the selectivity between these substances. The signal from the mixture of ascorbic acid and dopamine (Fig. 8b, trace 3) is virtually equal to that from neat 1 mM dopamine (Fig. 8b, trace 1). Importantly, the overall current from dopamine on coated and uncoated electrodes is virtually identical, i.e., the LBL coating does not decrease the detection limit of dopamine. The examination of the performance of the electrode at different ionic strengths demonstrates that the separation effect remains strong, even at the fairly high buffer concentration of 10 mM (Fig. 8c), improving slightly with decreasing ionic strength, as expected. It is important to note that the excellent performance of the coating for the physiological pH 7, and for different ionic strengths within the typical parameters of living organisms, makes possible the utilization of the nanoshell films in neurochemistry for the *in vivo* monitoring of neurotransmitters.

The LBL films made of PAA and PDDA displayed a significantly lower dopamine/ascorbic acid selectivity, with a selectivity enhancement in the range of 2–3 times greater than that of the native GC surface (Fig. 9). This proves the importance of the contribution of the nanoshell component in the nanoshell/PAA films to the ion-selectivity observed in Figure 8, and therefore of the nanoscale organization of charges to form the ionic channels in nanoshells. Although this is easier to attain with a rigid inorganic material, we do not want to discard the possibility of finding deposition conditions for polyelectrolyte/polyelectrolyte LBL films that might lead to a similar performance. However, such a task is beyond the scope of this paper.

The characterization of the electrochemical properties of the nanoshell coatings would be incomplete without looking into electrode poisoning. Unfortunately, the current quickly de-

creases with the number of scans, evidence for the formation of a film of by-products on the electrode surface (Fig. 10b). The same was observed for both PAA/PDDA-coated and native GC electrode surfaces (Figs. 10c and 10a, respectively). The data in Figure 10 also indicate that the loss of sensitivity pertains to the GC electrode rather than to the LBL coatings. Other electrode materials and/or deposition of a special (LBL) layer that prevents desensitization may help solve this problem.

3.4. Biocompatibility of LBL Films

The problem of electrode poisoning does not negate the possibility of establishing long-term operation of the coating in a living organism. First of all, the ion-selective properties of the nanoshells can be used for many other biomedical applications besides dopamine determination. Second, the biocompatibility of LBL films is a new and fairly complex property of such films that has not yet received proper attention. This necessitates the initial mapping out of the perspectives in this direction.

The biocompatibility of man-made materials derives from the interplay of many factors, with the basic requirements being the following:^[56,79,80] 1) the material must not be toxic to any living cell; 2) the material must possess chemical and physical properties that promote cell–substrate interactions for the tissue of interest; 3) materials for implantable devices must be relatively biologically inert, in order to avoid acute immune response of the organism.^[81]

Several facts point to favorable characteristics of the LBL nanoshell films as a biocompatible material. Firstly, one of the important parameters governing cell–substrate interactions is the surface roughness.^[80,82] Previously, it has been found that a surface topography with a roughness on the nanometer scale is preferable for cell attachment.^[82,83] The AFM images obtained (Fig. 4) show that, indeed, the surface roughness of the prepared films is in the desirable range. Secondly, porous structures have been found to support living cultures of mammalian tissues better than solid supports, because they facilitate the delivery of nutrients to the cells.^[83] This feature is also present in the prepared films. Thirdly, bulk titanium dioxide had been used for some biomedical applications, including acting as a support for growing cells^[84–86] and implant coating,^[87,88] which indicates that the chemical composition of at least one component of the LBL composite encourages cell growth. However, the question as to whether or not the biocompatibility of bulk TiO₂ is transferred to the nanoshells and to the LBL composite films still needs to be investigated.

The quality of the LBL nanoshell film as a biomaterial can be evaluated by the attachment of nerve cells. For this study, the latter are of particular importance since they are the primary source of neurotransmitters. A glass substrate with a nanoshell film was tested to determine whether the LBL films would support the growth of PC12 pheochromocytoma cells in a culture. These cells have the capacity to be further differentiated into neurons upon induction to a nerve growth factor. Surveying the surface with an inverted optical microscope

demonstrates that these cells successfully attach and grow on the prepared nanocomposite (Fig. 11). The cells attached in small clumps, and were initially distributed uniformly over the entire surface. Later, they formed tightly packed cell clusters, which displayed a tendency to merge with time. There was no sign of an adverse effect of the film component on the viability or growth of the PC12 nerve cell on the coating for several weeks. In fact, the PC12 cells appeared to attach more firmly to, and grow better on, the LBL films than on the commercial cell culture plates upon which they were originally grown.

Thus, these data demonstrate that the nanoshell LBL films satisfy at least two out of three of the basic requirements for biocompatibility. The study of the immune response of organisms to this new material entails testing it on live animals.

There was no attachment of PC12 culture cells to PAA/PDDA multilayers prepared in the same way. This could be related to the presence of the quaternary amine in PDDA, which is, in general, unfavorable for cell adhesion. The use of poly-(lysine) and/or a different layer architecture even for PDDA-containing films may significantly improve cell attachment.

4. Conclusion

A new type of biomaterial was prepared from TiO₂ nanoshells by using the LBL deposition procedure. As a result of their closely packed morphology and nanoscale dimensions of the pores inside the nanoshells, the TiO₂/PAA films displayed strong ion selectivity. This quality can be further optimized and tailored to various systems of interest by controlling the size and surface modification of the nanoparticles, owing to the exceptional versatility of the LBL deposition technique.

We utilized the ion-selective capabilities of the nanoshell films for the successful detection of dopamine in the presence of ascorbic acid, which represents an important neurochemical problem, and demonstrated a significant improvement in the detection selectivity, without any loss of sensitivity. The films were also found to be biocompatible with a nerve cell line, which opens the way for their exploitation as a coating on implantable electrodes for the in-vivo monitoring of brain activity. The ability to detect neurotransmitters directly inside the brain can significantly facilitate the understanding of chemical communication between neurons and the diagnoses of malfunctions of the nervous system. At the same time, the nanoshell/PAA coating did not demonstrate any improvement in terms of the poisoning of the GC electrode by the by-products of dopamine reduction. This necessitates the further search for a practically viable system for dopamine monitoring.

An important aspect of this study is that it presents a new example of the biomedical applications of nanoparticles. Considering that biocompatibility of the LBL films can easily be attained with an appropriate film sequence, the extension of the presented technique to other nanoparticle/polyelectrolyte films will enable the preparation of a large variety of biomaterials with different functionalities. In-vivo neurochemical monitoring is just one of a few possible areas where such films could be successfully utilized.

5. Experimental

Chemicals: All chemicals used in the synthesis of the nanoshells and film deposition were purchased from Sigma-Aldrich and were used without further purification. Ultrapure 18 MΩ/cm water was used for all experiments, as well as for glassware cleaning. Pheochromocytoma cells (PC12) were purchased from American Type Culture Collection (ATCC). The pH of the solutions was adjusted with diluted HCl or NaOH. All experiments were performed under atmospheric conditions, except for the electrochemical measurements (see below).

Preparation of TiO₂ Nanoshells: The precursor of the nanoshells, i.e., silver nanoparticles coated with a shell of titanium dioxide, were prepared by the combination of two processes that occur sequentially in a single reaction mixture—the formation of the silver core and the TiO₂ shell. This was achieved by hydrolyzing the appropriate metal alkoxide, Ti(OC₄H₉)₄, and simultaneously performing high-temperature reduction of Ag⁺ using a dimethylformamide (DMF)/ethanol mixture in the presence of acetylacetone [18]. A detailed description of the preparation procedure and the set-up is given elsewhere [18]. Briefly, the starting reaction mixture was prepared from two solutions. The first solution contained equimolar amounts of Ti(OC₄H₉)₄ and acetylacetone in ethanol, at a concentration of 5.75 mM for both components. Sonication in a Fisher Scientific FS20 ultrasound bath for 5 min was required to obtain a clear mixture. The second solution was 3.8 mM AgNO₃ and 0.8 M H₂O in DMF. Freshly prepared 20 mL of the first solution and 5 mL of the second solution were mixed in a round-bottom flask, and stirred while heating under reflux. After 90 min, the colloid attained a green-black color, indicating the formation of coated silver nanoparticles.

Titanium dioxide nanoshells were obtained by adding a 30 % solution of ammonium hydroxide to a solution of the original dispersion of nanoparticles in the ratio of 1:2. The silver core dissolved in ammonia as a result of its oxidation by atmospheric oxygen. In its ionic form, the silver core easily leaches from the pores in the TiO₂ shell of the particles into the water phase. The procedure was repeated three times (each time >3 h, with stirring) until complete bleaching of nanoparticles was attained. The oxidation of silver was monitored by UV-vis spectrophotometry by observing the gradual decrease of the silver surface plasmon band at 440 nm. After complete bleaching of the dispersion, the nanospheres were washed with ultrapure water and centrifuged three times, followed by redispersion at pH 2.5.

Layer-by-Layer Assembly: The films with the nanoparticle (NP) layers on different substrates (quartz, glass, silicon wafer, GC, and polystyrene cell culture plates) were constructed by LBL assembly with oppositely charged polyelectrolyte. They were cleaned with a freshly prepared “piranha” solution (a 2:1 mixture of concentrated H₂SO₄ and 30 % H₂O₂) for 5 min followed by extensive rinsing with water, and were finally dried under a stream of nitrogen. For the electrochemical experiments, a 3 mm diameter GC electrode (Cypress Inc.) was polished with a 0.05 μm alumina slurry.

PDDA, $M_w = 400\,000$ –500 000, adsorbed on the negatively charged surface of a substrate was the first layer for the LBL assembly. PAA, $M_w = 450\,000$, was used as the partner polyelectrolyte for TiO₂-on-Ag nanoparticles or TiO₂ nanoshells positively charged at low pH. The final assembly typically contained 20 layers of nanoparticles. After deposition on the substrate, the silver cores of the nanoparticles were removed from the multilayers by extraction with 30 % ammonium hydroxide, as described above. The samples were then rinsed with ultrapure water and dried in a stream of nitrogen. For electrochemical and other measurements, the LBL assembled films of both core-shell Ag/TiO₂ nanoparticles and TiO₂ nanoshells were employed.

Biocompatibility Study: PC12 cells for biocompatibility studies were cultured in RPMI 1640 medium (GIBCO) containing 5 % fetal bovine serum (FBS, Hyclone), 10 % horse serum (GIBCO), and 2 % streptomycin/penicillin (Sigma), at 37 °C in a CO₂/air (5 %:95 %) environment. The cells were removed from the culture dishes by pipetting with the medium. Before seeding, polystyrene dishes (Corning Inc.) with deposited LBL films were sterilized with 70 % ethanol and air-dried in a sterile hood. The cells, in fresh medium, were seeded on the substrates and allowed to adhere for 24 h (incubator, NuAir Inc.) and then grown with fresh media that was changed every 2–3 days. Cells were imaged using a Microflex UFX-DX microscope (Nikon Inc.) with 100× and 200× magnification.

Instrumentation: The size and shape of prepared nanoparticles were characterized by high-resolution TEM (a Phillips microscope operating at 120 kV). For the preparation of samples for electron microscopy, a drop of the nanoshell sol was placed onto a copper-coated carbon grid (200 mesh). The grid was allowed to dry for one minute, and then blotted dry with filter paper to remove the excess solution. The dried grids were transferred into a nitrogen-filled container and then to the cell compartment of the TEM microscope, equipped with a Phillips EDAX 9800 analyzer.

Surface imaging of the nanoparticle layers was performed by a Nanoscope IIIa Multimode surface probe microscope (Digital Instruments/Veeco). Atomic force microscopy and scanning tunneling microscopy images were obtained in tapping and constant current modes, respectively.

Ellipsometric measurements were made with an AutoEL MS ellipsometer from Rudolph Research Corp. The measurements were performed using the 632.8 nm line of a He/Ne laser incident upon the sample at 70°. The DafiBM program supplied by Rudolph Technologies was employed to determine the thickness values.

X-ray photoelectron spectra were recorded at a take-off angle of 0°, using a VG EscaLab 220-IXL system. The Cu Kα X-ray line [eV] of aluminum at a spectrometer pass energy of 1486.92 eV and a step resolution of 0.1 eV was used as the source.

Cyclic voltammetry experiments were carried out with a CV-50W BAS-50 B/V electrochemical analyzer system from Bioanalytical Inc. The three-electrode cell contained the GC working electrode, a platinum wire counter electrode, and an Ag/AgCl reference electrode. CV experiments were done at 20±0.5 °C. Solutions were deoxygenated by purging them with ultrapure nitrogen. The magnitude of the cathodic and anodic peaks was determined using electrochemical analyzer system software, by extrapolation to a base-line current.

Zeta-potential measurements were performed by using a Malvern Zetasizer 2000 HS operating with an internal 10 mW, 633 nm He–Ne laser in the right angle geometry. A standard 1×1 cm cuvette was used in these measurements.

Received: September 17, 2001
Final version: January 3, 2002

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